(18) Measurement of Neutrophil Elastase Activity with (N-Benzyloxycarbonyl-Ala-Ala)$_2$-Rhodamine 110

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Outline

Elastase is a lysosomal serine proteinase contained in large amounts in neutrophils, in lower amounts in monocytes, which degrades a large variety of biological substrates at neutral pH. Elastase is important for the degradation of bacteria. The extracellular release of elastase may lead to inflammatory tissue destruction in diseases such as sepsis, posttraumatic shock, myocardial reperfusion, or rheumatoid diseases. The nonfluorescent (N-benzyloxycarbonyl-Ala-Ala)$_2$-rhodamine 110 is intracellularly cleaved by elastase in a sequential way to the green fluorescent N-benzyloxycarbonyl-Ala-Ala-rhodamine 110 and free rhodamine 110. The specificity of the cellular fluorescence for elastase activity is shown by inhibition with the serine proteinase inhibitor DFP.

Specimen: 3 ml heparinized human blood (10 U heparin/ml)

Reagents

HBSS without phenol red or bicarbonate, supplemented with 10 mM HEPES (pH 7.35)

(N-benzyloxycarbonyl-Ala-Ala)$_2$-rhodamine 110 (MW 1028.79)
stock solution: 4 mM in DMF (4.12 mg/ml)

propidium iodide (PI) (MW 668.4)
stock solution: 3 mM (2 mg/ml) in 5 mM HEPES-buffered saline (0.15 M NaCl, pH 7.35)

specific inhibitor
diisopropylphosphofluoridate (DFP) (MW 184.15): 1 M in DMSO

Procedure

1. Layer 3 ml heparinized blood carefully on top of 3 ml lymphocyte separation medium. Allow erythrocytes to sediment for 40 minutes at room temperature.
2. Withdraw the upper 800 µl supernatant plasma and store on ice. This will contain platelets and approximately 2 x 10^7/ml unseparated leukocytes.
3. **For lysosomal elastase activity**, put 1.00 ml HBSS, 20 µl cell suspension, and 1 µl (N-benzyloxycarbonyl-Ala-Ala)$_2$-rhodamine 110 solution in a 12 x 75 mm polypropylene test tube (final (N-benzyloxycarbonyl-Ala-Ala)$_2$-rhodamine 110 concentration 4 µM). Incubate at 37°C. Take 250 µl aliquots at 10, 20, and 30 minutes.
4. **For specific inhibition of elastase activity**, put 1.00 ml HBSS, 20 µl cell suspension, and 1 µl DFP stock in a 12 x 75 mm polypropylene test tube. Cap the tube to avoid inhalation of DFP. Incubate for 10 minutes at 37°C. Add 1 µl (N-benzyloxycarbonyl-Ala-Ala)$_2$-rhodamine 110 solution. Continue incubation, taking 250 µl aliquots at 10, 20, and 30 minutes after addition.
5. Counterstain dead cells by incubating 250 µl stained cell suspension with 5 µl 3mM PI for 3 minutes on ice (final PI concentration 60 µM).
6. Run on flow cytometer. Afterwards, inactivate DFP-contaminated samples by transferral into 2 N NaOH.

Excitation: 488 nm (argon laser) or high pressure mercury arc lamp with 470-500 nm bandpass filter
Filters: 510-530 bandpass for rhodamine green fluorescence
600 nm longpass for PI (dead cells) red fluorescence

Reagents

HBSS	Sigma Chemical Co.	# H-1387
Propidium iodide	Serva	# 33671
Diisopropylphosphofluoridate	Aldrich Chemical Co.	# D12,600-4

(N-Benzyloxycarbonyl-Ala-Ala)$_2$-rhodamine 110 synthesized in analogy to Leytus *et al.*

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NOTES:

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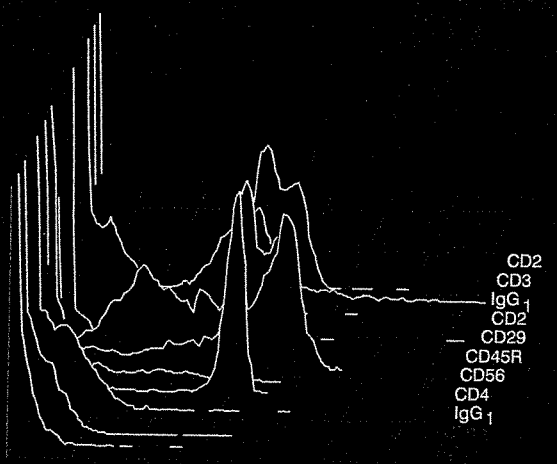

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